ClinGen Lysosomal Storage Disorders Variant Curation Expert Panel
Specifications to the ACMG/AMP Variant Interpretation Guidelines Version 2
This version specified for the following genes: *GAA*
Expert Panel Page: https://clinicalgenome.org/affiliation/50009/

Release notes:

1) Specifications for PS3 and BS3 have been revised and the strength has been downgraded.
2) PM2 has been downgraded to PM2_Supporting.
3) PP4 has been revised to allow the use of additional evidence types with strength of evidence based on a points system.
4) Cases are no longer required to meet the strict PP4 criterion in order to be counted for PM3.
5) Specifications for PM1 and BS2 are now included.
6) The tools used for in silico prediction of the impact of splice variants and in frame insertions and deletions for PP3 and BP4 have been revised.

PATHOGENIC CRITERIA		
Criteria	**Criteria Description**	**Specification**
VERY STRONG CRITERIA		
PVS1	Null variant in a gene where loss of function is a known mechanism of disease or in frame loss of an exon that contains residues involved in the active site of GAA. • Any nonsense, frameshift, or splice variant creating a premature stop codon before codon 916. • In frame deletions of an entire exon containing critical active site/substrate binding residues[a] (exons 8 and 10), or for which another variant removing the exon is known to be pathogenic (exons 2 and 18).	None
PM3_Very Strong	Detected in *trans* with a pathogenic variant. Points-based system. See main specifications document[b].	Strength
STRONG CRITERIA		
PS1	Same amino acid change as a previously established pathogenic variant regardless of nucleotide change.	None
PS2	*De novo (maternity and paternity confirmed) in a patient with the disease and no family history.*	*N/A*
PS3	Well-established in vitro or in vivo functional studies supportive of a damaging effect. • RT-PCR evidence of mis-splicing for non-canonical[c] intronic variants with no evidence of normal splice products.	None
PS4	*The prevalence of the variant in affected individuals is significantly increased compared to the prevalence in controls.*	*N/A*

PVS1_Strong	Null variant in a gene where loss of function is a known mechanism of disease. • In frame loss of an exon which is part of the catalytic barrel domain and contains pathogenic/likely pathogenic non-truncating variants (exons 6 and 9). • Initiator codon variant.	Strength, Disease-specific
PM3_Strong	Detected in *trans* with a pathogenic variant. Points-based system. See main specifications document[b].	Strength
MODERATE CRITERIA		
PM1	Located in a mutational hot spot and/or critical and well-established functional domain without benign variation • Missense substitution or in frame deletion of residues important in the active site architecture and substrate binding of GAA[a]:- D282, W376, D404, L405, I441, W481, W516, D518, M519, R600, W613, D616, W618, F649, L650, H674.	Disease-specific
PM2	Low frequency in population databases. • Minor allele frequency <0.1% (0.001) in all continental populations with >2000 alleles in gnomAD.	Disease-Specific
PM3	Detected in *trans* with a pathogenic variant. Points-based system. See main specifications document[b].	None
PM4	Protein length changes due to in-frame deletions/insertions in a non-repeat region or stop-loss variants. • In frame deletion/insertions of two or more amino acids but less than one exon.	None
PM5	Missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before.	None
PM6	*Confirmed de novo without confirmation of paternity and maternity.*	*N/A*
PVS1_Moderate	Null variant in a gene where loss of function is a known mechanism of disease. • Premature termination codon in the 3' end of *GAA* (3' to codon 916), not predicted to be detected by nonsense-mediated decay. • Predicted exon-skipping due to canonical splice variant or exon deletion resulting in an in frame deletion of <10% of the gene product (exons 17, 19, and 20).	Strength; Disease specific

PS3_Moderate	Well-established in vitro or in vivo functional studies supportive of a damaging effect. • <5% wild type GAA activity when the variant is expressed in a heterologous cell type and evidence of abnormal GAA synthesis and/or processing[d]. • RT-PCR evidence of mis-splicing for non-canonical intronic variants with evidence of normal splice products.	Strength; Disease specific
PP4_Moderate	Phenotype specific for disease with single genetic etiology. • Points-based system. See main specifications document[b].	Strength; Disease specific
SUPPORTING CRITERIA		
PP1	*Co-segregation with disease in multiple affected family members.*	*NA*
PP2	*Missense variant in a gene that has a low rate of benign missense variation and where missense variants are a common mechanism of disease.*	*N/A*
PP3	Multiple lines of computational evidence support a deleterious effect on the gene or gene product. • REVEL score >0.7 for missense variants. • In frame deletion or insertion predicted deleterious by 2 out of 3 tools (PROVEAN, MutationTaster, MutPred-InDel). • Predicted impact on splicing by SpliceAI (score >0.5).	Disease-specific
PP4	Phenotype specific for disease with single genetic etiology. • Points-based system. See main specifications document[b].	Disease-specific
PP5	*Reputable source recently reports variant as pathogenic but the evidence is not available to the laboratory to perform an independent evaluation*	*N/A*
PS3_Supporting	Well-established *in vitro* or *in vivo* functional studies supportive of a damaging effect. • <30% wild type GAA activity when the variant is expressed in a heterologous cell type[d]. • RT-PCR evidence of mis-splicing for non-canonical intronic variants with evidence of normal splice products.	Strength; Disease specific
PM3_Supporting	Detected in *trans* with a pathogenic variant. Points-based system. See main specifications document[b].	Strength
PM4_Supporting	Protein length changes due to in-frame deletions/insertions in a non-repeat region or stop-loss variants. • In frame deletion/insertions of one amino acid.	Strength
PM5_Supporting	Missense change at an amino acid residue where a different missense change determined to be “likely pathogenic” has been seen before.	Strength

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BENIGN CRITERIA		
Criteria	**Criteria Description**	**Specification**
STAND ALONE CRITERIA		
BA1	Common in population databases. • Highest minor allele frequency >0.01 (>1%) in any continental population in gnomAD with >2000 alleles.	Disease-Specific
STRONG CRITERIA		
BS1	Allele frequency greater than expected for disease. • Highest minor allele frequency >0.005 (>0.5%) in any continental population in gnomAD with >2000 alleles.	Disease-Specific
BS2	Observed in the homozygous state in a healthy adult. • Homozygous individual of any age with normal GAA activity.	N/A
BS3	*Well-established in vitro or in vivo functional studies show no damaging effect on protein function.*	*N/A (used at supporting strength)*
BS4	*Lack of segregation in affected members of a family.*	*N/A*
SUPPORTING CRITERIA		
BP1	*Missense variant in gene where only LOF causes disease*	*N/A*
BP2	Observed in *cis* with a pathogenic variant.	None
BP3	*In-frame deletions/insertions in a repetitive region without a known function*	*N/A*
BP4	Multiple lines of computational evidence suggest no impact on gene or gene product. • REVEL score <0.5 for missense variants. • In frame deletion or insertion predicted benign by PROVEAN, MutationTaster, and MutPred-InDel. • No predicted impact on splicing by SpliceAI (score <0.2)	Disease-specific
BP5	*Variant found in a case with an alternate molecular basis for disease.*	*N/A*
BP6	*Reputable source recently reports variant as benign but the evidence is not available to the laboratory to perform an independent evaluation.*	*N/A*
BP7	A synonymous (silent) variant for which splicing prediction algorithms predict no impact to the splice consensus sequence nor the creation of a new splice site AND the nucleotide is not highly conserved.	None

BS3_Supporting	Well-established *in vitro* or *in vivo* functional studies shows no damaging effect on protein function. • >50% activity when the variant is expressed in a heterologous cell type, or >30% activity if there is also evidence of normal synthesis and processing[d].	Strength; Disease-Specific

Disease-Specific: Disease-specific specifications are based on the currently available knowledge on GAA and Pompe disease; **Strength:** Increasing or decreasing strength of criterion based on the amount of evidence; **N/A:** not applicable for GAA and/or Pompe disease; **None:** no changes made to existing criteria definitions.

[a] Critical residues involved in active site architecture, mechanism, and/or substrate binding (see details in Hermans et al, 1991, PMID: 1856189; Deming et al, 2017; DOI 10.1101/212837; Roig-Zamboni et al, 2017, PMID: 29061980.
[b] Full specifications available at https://clinicalgenome.org/affiliation/50009/
[c] Non-canonical splice site variant refers to any variant that is not +1, +2, -2, -1.
[d] For example, see Kroos et al, 2008 (PMID 18425781), Kroos et al 2012 (PMID 22644586).

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General:

- Criteria will be combined as described in Richards et al, 2015.
- SpliceAI will be used to analyze all variants, including exonic variants such as missense, nonsense, and frameshift, for potential impact on splicing.
- If the variant is described only as an amino acid change in an evidence source, and the cDNA change is not provided, this is not sufficient and the evidence should not be used.

EVIDENCE OF PATHOGENICITY

PVS1: ***"Null variant (nonsense, frameshift, canonical ±1 or 2 splice sites, initiation codon, single or multi-exon deletion) in a gene where loss of function is a known mechanism of disease."***

LSD VCEP notes:

- Acid alpha-glucosidase is a monomeric lysosomal enzyme coded for by a single locus (*GAA*), with no evidence of alternative active isoforms. Loss of function (LoF) is a known mechanism for Pompe disease. There are numerous published examples of LoF variants (nonsense, frameshift, canonical +/-1 or 2 splice sites, initiation codon, single or multi-exon deletion) in *GAA* in individuals with Pompe disease. The specifications below, for assigning weight of evidence for LoF variants, are based on published guidance for assigning weight of evidence for PVS1 (Abou Tayoun et al, 2018, PMID 30192042).
- Additional considerations:
    - If PVS1 is applied, PM4 will not be applied.
    - If PVS1 is applied, in general, PS3 and PP3 will not be applied except for rare circumstances when approved by experts in the VCEP. However, the results of splicing assays and in silico prediction may be used to inform the strength of evidence for PVS1. For example, if a canonical splice site change is expected to result in exon skipping with an out of frame consequence but RT-PCR and in silico prediction data suggest that a cryptic splice site, resulting in an in frame consequence, is used the strength of PVS1 should be down-graded.
    - Curators are encouraged to record any data that supports the weight of evidence assigned for PVS1, and the application of this code.

***GAA* specifications:**

- **Nonsense and frameshift variants**
    - All nonsense and frameshift variants will meet PVS1 (Very Strong), unless the variant is predicted to be missed by nonsense-mediated decay (NMD) i.e. if the resulting premature termination codon is in the last exon (exon 20) or in the last 50 nucleotides of the penultimate exon (exon 19; after c.2749, codon 916). In this case, PVS1_Moderate will be applied because <10% of the primary amino acid sequence is predicted to be lost.
    - For frameshift variants at the 3' end of GAA that are not predicted to undergo NMD i.e. PTC downstream of c.2749, consider the length of abnormal amino acid sequence that is added due to the frameshift. If >10% of the length of the normal sequence is altered, PVS1 can be

applied at strong, but if <10% of the length of the normal sequence is altered, PVS1 can be applied at moderate.

- **Canonical splice site variants (+1, +2, -1, -2)**
  - All donor/acceptor splice sites in *GAA* follow the GT/AG rule, except for the donor splice site of intron 19 (the last intron) which begins with GC.
  - Variants in the donor splice junction of intron 19 will not meet PVS1 because the impact of alterations to GC donor splice sites is not well understood. PS3 could be applied if functional evidence is available. In silico splice site predictors should not be used for the donor splice site of intron 19 because they are designed for recognition of GT/AG splice sites.
  - For all variants involving either the +1 or +2 position of GT donor splice sites, the exon immediately 5' of the variant is predicted to be skipped. For all variants of either the -1 or -2 position of AG acceptor splice sites, the exon immediately 3' of the variant is predicted to be skipped. For the predicted in frame/out of frame consequences for skipping any exon in GAA, see Appendix 1.
  - Use SpliceAI in analysis of all canonical splice site variants (see PP3 and BP4 for details on thresholds). If there is a nearby (within +/- 20 nucleotides) splice site sequence that may reconstitute in-frame splicing, this should be taken into consideration.
  - Non +/- 1 or 2 canonical splice variants, such as +3 or -3, will not meet PVS1, but could meet PS3 and/or PP3 criteria.

- **Initiation codon**
  - All initiator codon variants will meet PVS1_Strong based on the observation that patients (n=3) homozygous for c.1A>G are CRIM-negative (Bali et al 2012, PMID 22252923; and personal communication). The next in-frame methionine is at position 122 but the likelihood of this start site being used is low and, even if used, the gene product would be missing the signal sequence (Bali et al, 2012, PMID 22252923).
  - While multiple initiator variants have been reported in *GAA*, PS1 or PM5 should not be used for these variants.

- **Deletions**
  - If a single or multi-exon deletion results in an out-of-frame consequence, use PVS1 (Very Strong) if NMD is predicted to occur. If NMD is not predicted to occur, use PVS1_Moderate.
  - If a deletion results in an in-frame consequence, the deletion must encompass one or more exons for PVS1 to apply. Consult Appendix 1 and use professional judgement regarding the strength of evidence to apply.
  - If an in-frame deletion is smaller than one exon, PVS1 does not apply; consider using PM4.

- **Duplications**
  - Single and multi-exon duplications have not yet been reported in *GAA*.
  - Use the PVS1 decision tree to assess the impact of single and multi-exon duplications.

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PS1: *"Same amino acid change as a previously established pathogenic variant regardless of nucleotide change. Caveats: Beware of changes that impact splicing rather than at the amino acid/protein level."*

***GAA* specifications:**

- This criterion is applicable as described.
- To avoid circularity, the classification of the other variant (Variant B) should not use evidence from the variant being interrogated (Variant A). If there is a question as to whether PS1 should be applied to variant A or variant B, use the classification of the variant with a greater level of evidence to support the classification of the variant with less evidence.

PS3: *"Well-established in vitro or in vivo functional studies supportive of a damaging effect on the gene or gene product. Note: Functional studies that have been validated and shown to be reproducible and robust in a clinical diagnostic laboratory setting are considered the most well-established."*

LSD VCEP notes:

- Data from any studies on samples from patients, such as GAA activity measurement in cultured skin fibroblasts or dried blood spots, is included in PP4 and should NOT be used for PS3.

***GAA* specifications:**

Any variant meeting the requirements below for either in *vitro* expression or splicing assays can meet PS3 (at the appropriate strength). If a variant meets the description for both e.g., a splice site variant with evidence of abnormal splicing and deficient GAA activity *in vitro*, PS3 must only be counted once but may be upgraded if both types of evidence are available.
Note that these assays are *in vitro*, research-based, and may not truly reflect in *vivo* function.

- ***In vitro* expression**
  There are several studies involving expression of GAA sequence variants in cultured cells and subsequent measurement of GAA activity. Some studies also include analysis of GAA synthesis and processing by Western blot and/or pulse chase, as well as immunolocalization studies. **Historically, these types of analyses have been broadly accepted when assessing the impact of a variant on GAA function**. Please see Appendix 2 for details on the methodology used in the studies listed below, and Moreland et al, 2005 (PMID 15520017) for details on GAA processing.

  - **Kroos et al, 2008 (PMID 18425781), Kroos et al 2012 (PMID 22644586), and additional studies from the Reuser group** – These studies were published by a group from Erasmus Medical Center in the Netherlands, led by Dr. Arnold Reuser. This group has decades of experience in the biochemical and molecular analysis of patients with Pompe disease, as well as the design and implementation of in vitro functional assays for GAA. In these studies, the use of Western blot analysis showing abnormal synthesis and/or processing of GAA supports the results obtained by the enzyme assay, and both types of data are incorporated into an overall "severity class". Based upon the expertise of the Erasmus group, the confirmation of findings from two assays

types (i.e. enzyme activity and Western blot), and agreement with results from functional analysis from other researchers when available, the results of these assays will be used at the moderate strength. The individual GAA activity and Western blot results for each variant are shown in Table 4 (PMID 18425781) and Tables 1A, 1B, and 1C (PMID 22644586).
    - PS3_Moderate will be applied to variants in severity Classes A and B, or the equivalent (i.e. GAA activity <5% and reduced mature, active GAA protein, which is represented by 76 and 70 kD bands) (PMIDs 18425781, 22644586) (see Appendix 2 for explanation)
    - PS3_Supporting will be applied to variants in severity classes C and D, or the equivalent (i.e. 5-30% GAA activity) (see Appendix 2 for explanation)

- **Flanagan et al (PMID 19862843) –** This study included expression of 76 different GAA variants in COS cells, measurement of enzyme activity (Figure 2, Table 2, Table 3), and display of Western blot and immunolocalization data for some variants.
    - PS3_Supporting will be applied to all variants in Table 2 (all have <15% WT activity) and Table 3 (all have <2% WT activity).

- Many additional papers have been published which include expression of GAA variants in a heterologous cell type followed by measurement of GAA activity, with results of Western blot, pulse chase, or immunolocalization analyses in some cases. As mentioned, **these assays are broadly accepted in the analysis of GAA variants as well as other enzymes involved in metabolic disease**.
  After assessment of the parameters listed below and discussion with the VCEP, results from such studies can be used at **PS3_Supporting for variants with <30% WT activity** if considered to be appropriate:
    - Were clones sequenced to verify that the variant is present and that no artifacts have been introduced during the site-directed mutagenesis process?
    - Were appropriate controls included? e.g.;
        - Negative controls: Empty vector, antisense (at least one appropriate negative control is required)
        - Positive control: Wild type GAA, normal cells (at least one appropriate positive control is required)
    - Was the experiment replicated?
    - If cells have intrinsic GAA activity e.g., COS cells, the level of activity should be stated so that this can be taken into account.

- **Splicing assays**
    - Apply PS3 for splicing assays (at the appropriate strength) only for <u>non</u> +/-1 or 2 splicing variants. For canonical +/- 1 or 2 splicing results of splicing assays should be taken into account and may inform the strength of evidence applied for PVS1.
    - PS3 can be applied for in vitro splicing assays such as mini-gene assay, RT-PCR, or RNA-Sequencing. These studies can be performed on mRNA extracted from patient-derived cells, or from heterologous cultured cells transfected with the variant. As results can be highly variable, caution and professional judgement should be used in their interpretation.

- For non +/-1 or 2 canonical splicing variants, use PS3 if there is RT-PCR and/or RNA sequencing evidence demonstrating only abnormal splice products, with no evidence of normal splicing. The impact of the splicing defect (inframe or out of frame; number of amino acids deleted/inserted) should be taken into account when deciding upon the weight of evidence applied. For example, an out of frame consequence could be weighted PS3, whereas use of a cryptic splice site resulting in a small insertion could be awarded PS3_Moderate.
- Note that evidence of normal and abnormal splicing may occur for patients who are compound heterozygotes for a splicing variant and another variant type that does not disrupt splicing. The presence of normal splice products can complicate the assessment of the impact of the splice variant. Therefore, consider downgrading to PS3_Moderate or PS3_Supporting if there is evidence of normal splice products, based on professional judgement.
- PP3 may also be used for non-canonical splice variants meeting PS3 at any strength.

PM1: *"Located in a mutational hot spot and/or critical and well-established functional domain (e.g., active site of an enzyme) without benign variation."*

LSD VCEP notes*:*
Based on the crystal structures of native GAA and rhGAA, the following residues are important in the active site of GAA - D282, W376, D404, L405, I441, W481, W516, D518, M519, R600, W613, D616, W618, F649, L650, H674 (Deming et al, 2017, https://www.biorxiv.org/content/10.1101/212837v1.full.pdf; Roig-Zamboni et al, 2017, PMID: 29061980). There are no benign or likely benign variants of these residues in ClinVar, Erasmus, or gnomAD (see Appendix 3). Of note, D518 is the catalytic nucleophile and D616 is the catalytic acid/base (Hermans et al, 1991, PMID: 1856189; Deming et al, 2017; Roig-Zamboni et al, 2017). The other residues are important in active site architecture and substrate binding.

***GAA* specifications:**
PM1 will be applied for any missense substitutions or inframe deletions of the above residues.

PM2: *"Absent from controls (or at extremely low frequency if recessive) in Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation Consortium AND not observed in the homozygous state."*

***GAA* specifications:**

- Any variant with a highest population minor allele frequency <0.1% (<0.001) in any continental population with >2000 alleles gnomAD will meet PM2_Supporting (see Appendix 4 for calculations). The weight of evidence is downgraded from PM2 to **PM2_supporting** based on guidance from the ClinGen SVI.

- Variants may be observed in the homozygous state because Pompe disease can present in adulthood, and some variants may be hypomorphic. However, the presence and number of homozygotes should be noted.
- There are examples of well-known pathogenic *GAA* variants with a higher frequency than 0.1% in gnomAD; the highest minor allele frequency of c.-32-13T>G, associated with late onset Pompe disease, is 0.005293 in European non-Finnish; the minor allele frequency of c.2560C>T (p.Arg854Ter), a well-known pathogenic infantile-onset, CRIM-negative allele, is 0.001891 in Africans; and the minor allele frequency of c.1935C>A (p.Asp645Asn), associated with infantile – onset Pompe disease in East Asians is 0.001729. However, as we assume that the most common pathogenic variants have already been identified, a more conservative cutoff was chosen for PM2_Supporting. There is ample evidence to classify these variants (c.-32-13T>G, p.Arg854Ter, and p.Asp645Asn) as pathogenic without using PM2_Supporting.

PM3: *"For recessive disorders, detected in trans with a pathogenic variant."*

***GAA* specifications:**

- To meet PM3, the patient must be described as having Pompe disease, at a minimum. If the case meets PP4 and PM3, both criteria are applied. However, our PP4 criteria need not necessarily be met in order to apply PM3, as long as the patient is stated to have Pompe disease.
- For rare variants that are routinely observed to be in cis with a pseudodeficiency variant, substantial additional evidence must be available to support the pathogenicity of the variant. The variant must meet PM2_Supporting, functional data should be available to support a deleterious impact, and cases considered for PM3 must have other clinical and laboratory findings supporting a diagnosis of Pompe disease. We strongly recommend not applying this exception for a novel variant with a single report.
- If multiple unrelated compound heterozygous cases have the same genotype, and the variants are not confirmed in trans, then no more than two cases should be used for assigning points (i.e. maximum of 1 point; similar to the guidance for homozygotes). This avoids over-counting evidence if the variants are actually in cis, and hence inherited together, in multiple individuals. Care must be taken to ensure that the reports do not represent the same case.
- Following SVI guidance for PM3 (https://clinicalgenome.org/site/assets/files/3717/svi_proposal_for_pm3_criterion_-_version_1.pdf), use the scoring system below to determine the strength of evidence for PM3. These variant interpretation guidelines should be used to determine the classification of the "other variant" in order to determine the appropriate number of points to assign.
- For a variant to be "confirmed in trans" in a compound heterozygous patient, parental testing in at least one parent, or another appropriate molecular method (such as cloning each allele separately followed by sequencing), must have been performed. Otherwise, the phase of the variants is unknown. Parental testing is not required for homozygous cases.
- As noted in the SVI guidance for PM3, "To avoid circularity, in all instances (phasing confirmed or unknown), the classification of the variant on the other allele should not use evidence from the variant being interrogated."

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Classification/Zygosity of other variant	***Points per Proband***	
	Confirmed in trans	***Phase unknown***
Pathogenic or Likely pathogenic variant	*1.0*	*0.5 (P)* *0.25 (LP)*
Homozygous occurrence (max points = 1.0)	*0.5*	*N/A*

PM3 Point Table			
PM3_Supporting	**PM3**	**PM3_Strong**	**PM3_VeryStrong**
0.5 points	**1.0 points**	**2.0 points**	**4.0 points**

PM4: *"Protein length changes as a result of in-frame deletions/insertions in a non-repeat region or stop-loss variants."*

- **LSD VCEP notes:**
  - In-frame deletions and insertions have been reported in *GAA*.
  - Stop loss has not been reported in *GAA*, as far as we are aware, other than as a result of frameshift variants.

***GAA* specification**

- Downgrade to PM4_Supporting for single amino acid deletions and insertions. Otherwise, use this rule "as is".
- For in-frame deletions of one or more exons, use PVS1.

PM5: *"Novel missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before. Caveat: Beware of changes that impact splicing rather than at the amino acid/protein level."*

***GAA* specification:**

- If the pathogenicity of another missense change at the same amino acid residue is unknown, determine its pathogenicity using these guidelines in order to determine if this criterion can be used. If the other variant is Pathogenic, use PM5. If the other variant is Likely Pathogenic, use PM5_Supporting.

- To avoid circularity, the classification of the other variant (variant B) should not use evidence from the variant being interrogated (variant A). If there is a question as to whether PM5 should be applied to variant A or variant B, use the classification of the variant with a greater level of evidence to support the classification of the variant with less evidence.

PP3: *"Multiple lines of computational evidence support a deleterious effect on the gene or gene product (conservation, evolutionary, splicing impact, etc.)"*

GAA specification:

- Any missense changes with a REVEL score >0.7 will meet PP3 (see Appendix 5 for data used to determine the threshold).
- For in frame insertions and deletions, use PROVEAN (http://provean.jcvi.org/seq_submit.php (score <-2.5 for "deleterious"), Mutation Taster (http://www.mutationtaster.org/ (count if "disease-causing"), and MutPred-Indel (http://mutpredindel.cs.indiana.edu/ (score >0.5 for "pathogenic"). Apply PP3 if two of the three predictors indicate that the variant is deleterious.
- For non-canonical splice site variants (e.g., +3, -3), use SpliceAI (https://spliceailookup.broadinstitute.org/). A score of >0.5 is taken to indicate disruption of the splice site allowing PP3 to be applied. Evidence for use of a cryptic splice site and the impact on the gene product should also be assessed.

PP4: ***"Patient's phenotype or family history is highly specific for a disease with a single genetic etiology."***

LSD VCEP notes

Because the evidence to support a diagnosis of Pompe disease in the published literature varies according to the types of evidence presented and the level of detail provided, a points scheme for determining weight of evidence for PP4 will be used.

***GAA* specification**

Points will be awarded for each piece of evidence for a specific patient (see table).
For a total of 2 points or more, PP4_Moderate will be applied; for 1 point, PP4 will be applied.

- In order to count any of the evidence below, the authors must also state that the patient has Pompe disease.
- Do not apply this rule if the variant meets BA1, or otherwise meets criteria for benign or likely benign status.
- If either of the pseudodeficiency variants c.1726G>A (p.Gly576Ser) or c.2065G>A (p.Glu689Lys) are present, whether heterozygous or homozygous, deficiency of GAA activity cannot be used to apply PP4. If c.271G>A (p.Asp91Asn) is present, deficiency of GAA activity cannot be used to apply PP4 if glycogen was the assay substrate, but the data can be used if 4-MU was the substrate (Niño et al, 2020; PMID 33162552).

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Description of evidence	Points
Deficient GAA activity[a], documented as either 1) <10% of normal mean control level of GAA activity in leukocytes, lymphocytes, or muscle[b] samples, and/or <30% in cultured fibroblasts[c], or 2) Activity in the affected range (which must be provided in the publication) in any appropriate tissue (muscle, cultured skin fibroblasts, leukocytes, lymphocytes, whole blood or dried blood spot).	2
Patient reported to have Infantile Onset Pompe disease (IOPD) AND documentation of symptoms[d] of that condition. At a minimum, cardiomegaly, hypertrophic cardiomyopathy, left ventricular hypertrophy or a related term, and hypotonia, muscle weakness, or a related term, must be reported.	1
Cross reactive immunological material (CRIM) study of cultured skin fibroblasts or peripheral blood mononuclear cells reported to show absence of the 76 Da and 70 kDa bands, which represent the mature, active GAA enzyme[e]. This includes patients described as CRIM-negative (with no detectable GAA protein on Western blot), or those who are CRIM-positive but do not make the mature protein (e.g. only 110 kDa and 95 kDa bands are present).	1
The patient is reported to be on enzyme replacement therapy[f] for Pompe disease.	1
GAA activity is reported to be deficient[a] but the data are not provided (i.e. values for the patient and normal range as determined by the testing laboratory)	0.5
Patient identified as affected by newborn screening.	0.25
Urinary Glc_4[g] is elevated above the normal range.	0.25
Muscle MRI shows evidence of Pompe disease.	0.25
Muscle histology[h] is consistent with Pompe disease; there is glycogen storage in the lysosomes of muscle cells appearing as vacuoles that stain positively with periodic acid-Schiff.	0.25

Notes:

[a] If either of the known either of the pseudodeficiency variants c.1726G>A (p.Gly576Ser) or c.2065G>A (p.Glu689Lys) are present, whether heterozygous or homozygous, deficiency of GAA activity cannot be used to apply PP4. If c.271G>A (p.Asp91Asn) is present, deficiency of GAA activity cannot be used to apply PP4 if glycogen was the assay substrate, but the data can be used if 4-MU was the substrate (Niño et al, 2020; PMID 33162552).

[b] <10% activity in muscle is used because activity of GAA in muscle samples can overlap in patients with LOPD and carriers.

[c] To account for higher residual GAA activity in patients with late onset Pompe disease (LOPD)
[d] Symptoms in patients with IOPD are fairly specific with few other conditions mimicking this disorder. Therefore, documentation of symptoms is not considered to be sufficient for application of PP4
[e] For further details on the synthesis and intracellular processing and transport of GAA, and CRIM analysis in patients with Pompe disease, please see Moreland et al, 2005, PMID 15520017; Bali et al, 2012; PMID 22252923; Bali et al, 2015, PMID 26693141.
[f] If a patient is receiving Enzyme Replacement Therapy, the assumption is that their diagnosis of Pompe disease is well supported by clinical and laboratory evaluations.
[g] Elevated urinary glucose tetrasaccharide (Glc_4, also known as hexose tetrasaccharide or Hex_4), which is derived from the amylolytic degradation of glycogen, has a high sensitivity and specificity for Pompe disease (Young et al, 2012, PMID 22252961; Piraud et al, 2020, PMID 32382504 and references therein)
[h] While histochemical evidence of glycogen storage in muscle is supportive of a glycogen storage disorder it is not specific for Pompe disease.

PATHOGENIC RULES NOT USED

PS2: *"De novo (both maternity and paternity confirmed) in a patient with the disease and no family history. Note: Confirmation of paternity only is insufficient. Egg donation, surrogate motherhood, errors in embryo transfer, and so on, can contribute to non-maternity."*

LSD VCEP notes:

- D*e novo* variants are rarely reported in *GAA* (PMIDs 7981676, 27142047). The occurrence of *de novo* variants in GAA is not a mechanism of disease for Pompe disease, and the observation that a variant in *GAA* has arisen de novo does not support its causality. Any *de novo* variants will be assessed based on the variant type, functional evidence, and in trans data as described in these guidelines.

PS4: *"The prevalence of the variant in affected individuals is significantly increased compared with the prevalence in controls."*

LSD VCEP notes:

- There are no case-control studies for Pompe disease. As this is a recessive disorder, the prevalence of the variant in affected individuals may not be increased compared to controls (who could be heterozygous carriers). The number of patients with the variant will be addressed by the PM3 evidence code.

PM6: *"Assumed de novo but without confirmation of paternity and maternity."*

LSD VCEP notes:

- See explanation for PS2.

PP1: *"Cosegregation with disease in multiple affected family members in a gene definitively known to cause the disease."*

LSD VCEP notes:

- Sib-ships large enough to meet this criterion are extremely rare. In addition, because *GAA* is the only gene involved in Pompe disease, all patients are expected to be bi-allelic, regardless of whether the pathogenic variants can be, or have been, detected. A variant under assessment may not be the true pathogenic variant but instead in linkage disequilibrium with an unidentified pathogenic variant. For this reason, this criterion does not facilitate assessment of pathogenicity.

PP2: *"Missense variant in a gene that has a low rate of benign missense variation and in which missense variants are a common mechanism of disease."*

LSD VCEP notes:

- Does not apply; there are benign and pathogenic missense variants in *GAA.*

PP5: *"Reputable source recently reports variant as pathogenic, but the evidence is not available to the laboratory to perform an independent evaluation."*

LSD VCEP notes:

- Per SVI recommendation.

EVIDENCE OF BENIGN IMPACT See end of list for rules not used

BA1: *"Allele frequency is >5% in Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation Consortium."*

***GAA* specifications:**

- Any variant with the highest minor allele frequency >0.01 (>1%) in any continental population in gnomAD with >2000 alleles. Continental population = European non-Finnish, African, East Asian, South Asian, and Latino (Ghosh et al, 2018, PMID 30311383).
- See calculations in Appendix 4.

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BS1: *"Allele frequency is greater than expected for disorder."*

***GAA* specifications:**

- Any variant, other than c.-32-13T>G, with the highest minor allele frequency >0.005 (>0.5%) in any continental population in gnomAD with >2000 alleles. Continental population = European non-Finnish, African, East Asian, South Asian, Latino (Ghosh et al, 2018, PMID 30311383).
- Note that the highest allele frequency of the most common known pathogenic variant in GAA, c.-32-13T>G, is 0.00533 (0.53%) in European non-Finnish. This variant is exempted from meeting the BS1 criterion because it is the most common pathogenic variant in patients with late onset Pompe disease (Kroos et al, 2012, PMID 22253258)
- See calculations in Appendix 4.

BS2: *"Observed in a healthy adult individual for a recessive (homozygous), or dominant (heterozygous), or X-linked (hemizygous) disorder with full penetrance at an early age."*

***GAA* specifications:**

- BS2 can be applied if there is clear documentation that an individual of any age is homozygous for the variant has normal GAA activity. Values for GAA activity and the reference range for the laboratory must be provided.
- Patients with late onset Pompe disease can present late in life (5^{th}-6^{th} decade), can have mild symptoms, and may remain undiagnosed. Therefore, it is possible that homozygotes for hypomorphic GAA variants could be present in population databases

BS3: ***"Well-established in vitro or in vivo functional studies show no damaging effect on protein function or splicing."***

***GAA* specifications:** The same assays outlined for PS3 will be used for BS3. Please see PS3 guidance for additional information on these assays. BS3 will be applied as noted below:

- BS3_Supporting will be applied to any variant with >50% activity, when expressed in a heterologous cell type. If evidence of normal synthesis and processing is available, variants with >30% activity can meet BS3_Supporting. This includes Class E and F variants in Kroos et al, 2008 (PMID 18425781) and Kroos et al 2012 (PMID 22644586).

BP2: *"Observed in trans with a pathogenic variant for a fully penetrant dominant gene/disorder or observed in cis with a pathogenic variant in any inheritance pattern."*

***GAA* specifications:**

- Observed in *cis* with a pathogenic variant (to take autosomal recessive inheritance into account).

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BP4: *"Multiple lines of computational evidence suggest no impact on gene or gene product (conservation, evolutionary, splicing impact, etc.). Caveat: Because many in silico algorithms use the same or very similar input for their predictions, each algorithm cannot be counted as an independent criterion. BP4 can be used only once in any evaluation of a variant."*

***GAA* specifications:**

- Missense changes with a REVEL score <0.5 (see Appendix 5 for data used to determine the threshold).
- For in frame insertions and deletions, use PROVEAN (http://provean.jcvi.org/seq_submit.php (score >-2.5), Mutation Taster (http://www.mutationtaster.org/ (count if "polymorphism"), and MutPred-Indel (http://mutpredindel.cs.indiana.edu/ (score <0.5). Apply BP4 if all predictors indicate that the variant is benign.
- For non-canonical splice site variants, use SpliceAI (https://spliceailookup.broadinstitute.org/) to assess the impact of variants that are not +/-1 or 2 canonical splice site variants. This criterion can be applied for SpliceAI scores <0.2. If there is evidence for possible creation of a cryptic splice site, this criterion should not be applied.
- If there is any evidence for possible creation of a cryptic splice site, this criterion should not be applied.

BP7: *"A synonymous (silent) variant for which splicing prediction algorithms predict no impact to the splice consensus sequence nor the creation of a new splice site AND the nucleotide is not highly conserved."* (used as is; no modifications/specifications)

***GAA* specifications:**

- Apply this criterion as described.
- PhyloP score <0.1, or the variant is the reference nucleotide in 1 primate and/or 3 mammalian species.

RULES NOT USED

BS4: *"Lack of segregation in a family. Caveat: The presence of phenocopies for common phenotypes."*

BP1: *"Missense variant in a gene for which primarily truncating variants are known to cause disease."*

LSD VCEP notes:

- Does not apply. All types of variants cause Pompe disease.

BP3: *"In-frame deletions/insertions in a repetitive region without a known function."*

LSD VCEP notes:

- There are no known repetitive regions without known function in *GAA*.

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BP5: *"Variant found in a case with an alternate molecular basis for disease."*

LSD VCEP notes:

- An individual could be a carrier of a pathogenic variant in *GAA* and have another disorder.
- There is no known alternate molecular basis for deficiency of GAA activity, other than variants in *GAA*.

BP6: *"Reputable source recently reports variant as benign, but the evidence is not available to the laboratory to perform an independent evaluation."*

LSD VCEP notes:

- Per SVI recommendation.

Flowchart providing weight of evidence for PVS1 for GAA (based on Figure 1, Abou Tayoun et al, 2018, PMID 30192042)

Type of variant	Molecular consequence [a]		PVS1 strength
Nonsense	Predicted to undergo NMD		PVS1
	Not predicted to undergo NMD [b]		PVS1_Moderate [c]
Frameshift	Predicted to undergo NMD		PVS1
	Not predicted to undergo NMD[b]	>10% of the length of the normal sequence is altered	PVS1_Strong
		<10% of the length of the normal sequence is altered	PVS1_Moderate
GT-AG +/-1,2 splice sites[d]	Exon skipping[e] or use of a cryptic splice site disrupts reading frame and is predicted to undergo NMD	Exons 3 - 5, 7, 11-16[f]	PVS1
	Exon skipping[e] or use of a cryptic splice site disrupts reading frame and is not predicted to undergo NMD[b]	>10% of the length of the normal sequence is altered	PVS1_Strong
		<10% of the length of the normal sequence is altered	PVS1_Moderate
	Exon skipping[e] or use of a cryptic splice site preserves reading frame	Exons 2, 8, 10, 18[f]	PVS1
		Exons 6, 9[f]	PVS1_Strong
		Exons 17, 19, 20[f]	PVS1_Moderate
Initiation codon[g]			PVS1_Strong
Deletion	Full gene deletion		PVS1
	Single to multi-exon deletion disrupts reading frame and is predicted to undergo NMD		PVS1
	Single to multi-exon deletion disrupts reading frame and is not predicted to undergo NMD [b]	>10% of the length of the normal sequence is altered	PVS1_Strong
		<10% of the length of the normal sequence is altered	PVS1_Moderate
	Single to multi-exon deletion preserves reading frame	Consult Appendix 1[f]	Apply PVS1 at highest possible strength depending upon which exon(s) is/are deleted
Duplication	Proven in tandem	Reading frame disrupted and NMD predicted to occur	PVS1
		No known impact on reading frame and NMD	N/A
	Presumed in tandem	Reading frame presumed disrupted and NMD predicted to occur	PVS1_Strong
		No known impact on reading frame and NMD	N/A
	Proven not in tandem		N/A

Footnotes

[a] In GAA, all exons are biologically relevant; there is no significant alternative splicing.

[b] Not predicted to undergo NMD if the PTC is in the last exon (exon 20) or in the last 50 nucleotides of the penultimate exon (exon 19, 3' to c.2749, codon 916).

[c] In this case, <10% of the primary amino acid sequence is predicted to be lost.

[d] All donor/acceptor splice sites in GAA follow the GT/AG rule, except for the donor splice site of intron 19 (the last intron) which begins with GC. Variants in the donor splice junction of intron 19 will not meet PVS1.

[e] If the GT donor splice site is altered, it is predicted that the upstream exon will be skipped. If the AG acceptor splice site is altered, it is predicted that the downstream exon is skipped. This prediction can be overridden by experimental data, such as RT-PCR. The prediction can also be overridden by in silico prediction, but only if the in silico prediction is less deleterious than the predicted consequence.

[f] See Appendix 1 for details on the length of exons, and critical residues within exons. Note that exon 1 is untranslated.

[g] Patient (n=3) homozygous for c.1A>G are CRIM-negative (Bali et al 2012, PMID 22252923; and personal communication). The next in-frame methionine is at position 122 but the likelihood of this start site being used is low and, even if used, the gene product would be missing the signal sequence (Bali et al, 2012, PMID 22252923).